VIROLOGY 214, 59–71 (1995)

Biochemical and Genetic Analyses of the Interaction between the Helicase-like

ERIN K. O'REILLY,* NIANJUN TANG,* PAUL AHLQUIST,† and CHRIS CHENG KAO*,[1]

Department of Biology, Indiana University, Bloomington, Indiana 47405; and †Institute for Molecular Virology and Department of Plant Pathology, University of Wisconsin, Madison, Wisconsin 53706-1596

Replication of the three positive-strand genomic RNAs of brome mosaic virus requires the activities of the helicase-like 1a and the polymerase-like 2a proteins. One hundred fifteen amino acids of the 2a N-terminus and the 1a helicase-like region of over 50 kDa are both necessary and sufficient for 1a–2a interaction. Requirement of the large size of the 1a helicase-like domain suggests that higher order structures might be necessary for the protein's interaction with 2a. To explore the structural properties of 1a, we used limited proteolysis of *in vitro*-translated 1a protein. Treatment of 1a and its deletion derivatives with papain or trypsin revealed that the C-terminal helicase-like segment of approximately 50–60 kDa is highly resistant under our assay conditions to proteolysis, while the N-terminus is rapidly degraded. All tested mutations in the helicase-like region that renders this region protease-sensitive have previously been found to be defective for RNA replication *in vivo.* To complement the *in vitro* studies, we examined the interaction of the 1a helicase-like domain and the 2a N-terminus in yeast using the two-hybrid system. Mutations previously known to disrupt 1a–2a interaction also prevented interaction in yeast. Furthermore, results from two-hybrid analysis suggest that the structural domain mapped *in vitro* is important for 1a–2a interaction. Finally, we found that the helicase-like proteins of three other tripartite RNA viruses also contain equivalently located protease-resistant domains.

INTRODUCTION

The bromoviruses, cucumoviruses, and ilarviruses are plant virus groups within the alphavirus-like superfamily (Goldbach *et al.,* 1991). The viruses in these three genera are similar in many ways. They have tripartite genomes composed of capped, messenger-sense RNA and, like other alphavirus-like viruses, they have membrane-associated replicase complexes that include substantially conserved virus-encoded polymerase-, helicase-, and methyltransferase-like proteins (Argos, 1988; Haseloff *et al.,* 1984; Kamer and Argos, 1984).

Brome mosaic virus (BMV), the type member of bromoviruses, infects grasses including barley and wheat (Lane, 1981). BMV has proved to be a useful model system for studying the gene function and replication of positive-strand RNA viruses (Ahlquist, 1992). The BMV genome is composed of three genomic RNAs named RNAs 1, 2, and 3. RNAs 1 and 2 encode the two replication proteins, the 109-kDa 1a and the 94-kDa 2a, respectively (Ahlquist, 1992). Expression of 1a and 2a is sufficient for the replication of BMV RNAs in plant protoplasts (Dinant *et al.,* 1993; French *et al.,* 1986; Kiberstis *et al.,* 1981).

Several domains have been identified in BMV 1a and 2a based on sequence comparisons among viral replication proteins. The 1a polypeptide is composed of two conserved domains that are separated by a possible hinge region (Kao and Ahlquist, 1992). The N-terminal domain has sequence homology to the Sindbis virus nsP1 methyltransferase protein, which is believed to be involved in capping of viral RNAs (Haseloff *et al.,* 1984; Mi and Stollar, 1991; Scheidel and Stollar, 1991). The C-terminal portion of the 1a polypeptide has homology to viral and cellular helicases (Evans *et al.,* 1985; Gorbalenya *et al.,* 1988). Mutations in either the putative capping or the helicase domains can abolish RNA replication in transfected barley protoplasts (Kroner *et al.,* 1990). The 2a polypeptide consists of a highly conserved central polymerase-like domain flanked by sequences with a lower degree of conservation. Mutations in the polymerase-like domain abolish RNA replication, while some deletions in the flanking domains have only minor effects on replication in protoplasts (Traynor *et al.,* 1991).

The 1a and 2a proteins are components of an enzyme complex capable of negative-strand synthesis *in vitro*. The two proteins copurify in enzymatically active RNA-dependent RNA polymerase fractions from BMV-infected barley (Kao *et al.,* 1992; Quadt *et al.,* 1988). Several approaches were taken to demonstrate that 1a and 2a interact (Quadt and Jaspars, 1990; Kao *et al.,* 1992) and that this interaction is necessary for at least some steps of RNA replication (Kao and Ahlquist, 1992; Traynor *et al.,* 1991). The interacting regions have been mapped to the helicase-like domain of 1a and the N-terminal noncon-

[1] To whom correspondence should be addressed. FAX: (812) 855-6705. e-mail: ckao@sunflower.bio.indiana.edu.

0042-6822/95 $12.00

served portion of 2a (Kao and Ahlquist, 1992). These experiments revealed that some truncated forms of both 1a and 2a were able to form specific protein–protein complexes, suggesting that stable, binding-competent structures exist within some truncated 1a and 2a molecules. In order to probe further the interaction of the BMV RNA replication proteins, we used an *in vitro* limited proteolysis assay and the yeast two-hybrid assay.

MATERIALS AND METHODS

Plasmids and viral cDNAs

The BMV 1a and 2a plasmids used and the names of their encoded proteins are described in Tables 1 and 2, respectively. The AlMV RNA1 and RNA2 cDNAs, pUT17S (Cornelissen *et al.,* 1983a), and pUT27S (Cornelissen *et al.,* 1983b), respectively, were kind gifts of J. Bol (Leiden University, Netherlands). The CMV RNA1 and RNA2 were the Fast New York strain, cloned as pFny106 (Rizzo and Palukaitis, 1989) and pFny206 (Rizzo and Palukaitis, 1988), respectively, and were the kind gifts of P. Palukaitis (Cornell University, NY). The CCMV clones were previously described by Allison *et al*. (1988) and Dzianott and Bujarski (1991).

General molecular techniques

Polypeptides used for the protease digestion were synthesized from plasmids using a coupled bacteriophage T7 transcription and rabbit reticulocyte lysate translation extract (TNT kit, Promega) and labeled with [^{35}S]methionine (Amersham). Plasmid DNAs used for cloning and gene expression were initially purified through Qiagen columns (Qiagen Inc.), but were further extracted with a 1:1 mixture of phenol and chloroform and precipitated with ethanol. Polymerase chain reactions (PCR) were performed for 30 cycles, with each cycle consisting of denaturation at 94° for 1 min., annealing at 45 to 50° for 0.5 min., and elongation at 72° for 2 min. PCR-generated fragments were first cloned into pCR1000 (Invitrogen) and then digested with restriction enzymes for cloning into appropriate plasmids.

Protease digestions

Protease treatment of *in vitro*-translated proteins was primarily performed with papain according to the protocol of Pabo *et al*. (1979). A standard assay reaction used 2.5 Tl of *in vitro* translation extract mixed with an equal volume of 2× B buffer (1× concentration: 200 m*M* KCl, 10 m*M* Tris–Cl, pH 8.0, 2 m*M* $CaCl_2$, 2 m*M* cysteine, 0.5 m*M* ethylenediaminetetrachloric acid, 0.1 m*M* dithiothreitol, and 5% glycerol) containing 100 ng of papain that had been freshly diluted in B buffer from a stock solution at 60 mg/ml. The stock solution of papain was stored in B buffer at 4° for up to 2 months. The digestions were carried out at 30° unless noted otherwise and were terminated by pipeting the mixture into 15 Tl of 2× Laemmli sample buffer [1×: 62.5 m*M* Tris–CL, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.715 *M* B-mercaptoethanol, 0.04% bromophenol blue (Laemmli, 1970)] and frozen on dry ice until electrophoresis. Trypsin digestions were performed with 5 ng of trypsin (Sigma) in T buffer (20 m*M* $CaCl_2$, and 20 m*M* Tris–Cl, pH 8.0). The stock solution of trypsin, at 10 mg/ml, was stored at −20° in T buffer amended with 25% glycerol.

Electrophoresis was performed in polyacrylamide–SDS gels (SDS–PAGE) according to the protocol of Laemmli (1970). Afterward the gels were fixed in a solution of 20% acetic acid and 20% methanol for 30 min followed by a 10-min wash in deionized water and a 20-min soak in 1 *M* sodium salicylate. The gels were then dried and exposed to X-ray film (Fuji Photo Film Co.) at room temperature.

Construction of plasmids encoding truncated polypeptides

DNA fragments encoding BMV 1a polypeptides that have deletions of amino acids 2–517, 2–556, and 2–567 were generated by PCR. The 5′ oligonucleotide primers all contained a *Bam*HI site and three extra nucleotides at the 5′ end to facilitate cloning. The primers, with the viral sequences underlined, were: 1aΔ517 (5′ATAGGATCCATCATGGCTGAGATACCTCAGG3′), 1aΔ556 (5′ATAGGATCCATCATGGTACCAACGGACCCTCGTG3′), and 1aΔ567 (5′ATAGGATCCATCATGGGAGCCATGAAGGAATTTG3′). The 3′ primer (1a3′orfHIII, 5′ATAAAGCTTTCATCACTTAACACAATTAAAG3′), containing a *Hin*dIII restriction site and annealing to 1a nucleotides 2942 to 2962, was used in combination with all three 5′ primers in PCR reactions. The PCR-generated fragments were digested with *Bam*HI and *Hin*dIII and cloned behind the bacteriophage T7 promoter in plasmid pBS+ (Stratagene).

A DNA fragment encoding the CCMV 1a protein deleted from residues 2 to 547 was made using PCR with a 5′ primer (CKC1: 5′ATAAGATCTATGGTGACCGTTGGAGCTGAACCA3′) that annealed to codons 548–554 of the 1a gene and contained a unique *Bgl*II site. The 3′ primer (CKC2: 5′GCATGCCTGCAGGTCGACTC3′) contained a *Pst*I site that hybridized to sequences between noncoding nucleotides 3196 and 3176 of CCMV cDNA clone pCC1TP1 (Allison *et al.,* 1988). The DNA fragment was digested with *Bgl*II and *Pst*I and cloned into the compatible *Bam*HI and *Pst*I sites in pBS+.

Restriction fragments encoding a deletion of AlMV P1 amino acids 2–520 and 2–675 were generated with 5′ primers that contained *Bam*HI sites and the underlined AlMV sequences: CKA1 (5′ATAGGATCCATCATGGGTGTCTTTTACCCTATAATAAGG3′) and CKA2 (5′ATAGGA-

TCCATCATGGCAATTCCAACGCCAAAACCG3′). The 3′ primer (CKA3: 5′ATACTGCAGCCTTAGGGGCATTCATGCAA3′) annealed 3′ of the P1 coding sequence at nucleotides 3619 to 3642 and contained a *Pst*I site. It was used in combination with either CKA1 or CKA2 in PCR reactions. The resulting products were digested with *Bam*HI and *Pst*I and cloned into pBS+.

Construction of two-hybrid vectors

The two-hybrid vectors used were those previously described by Chien *et al.* (1991). The N-terminal 140 amino acids of the 2a polymerase-like protein were fused to the GAL4 transcription activation domain and cloned as a *Hin*dIII fragment in plasmid pAAH5. The orientation of the DNA encoding 2a was determined by restriction analysis. DNA fragments encoding deletions of 2a amino acids 2–40, 2–70, and 2–90 were made by PCR using the same 3′ oligonucleotide, 5′AGGATCCCTATAGATCTCTCGCATGATCTTC3′ (underlined), and the following 5′ oligonucleotides (viral sequences underlined): Δ2-40 (5′AGAATTCGGAGTTGCCATTGACG3′), Δ2-70 (5′AGAATTCCGAGTGCGCTGGGGCTC3′); and Δ2-90 (5′AGAATTCAGACCGCTGATTCCTCA3′). PCR fragments were cloned into plasmid pGAD424 behind the GAL4 transcription activation domain. Truncations of the 1a helicase-like domain were generated by PCR and cloned into pBTM116 behind the LexA sequence. The 3′ primer, B102 (5′ATAGGATCCTCACTCAGAGACAAGCG3′), was identical for all PCR reactions and contained a *Bam*HI site for cloning. The 5′ primers were a nested series which created N-terminal deletions up to the amino acid stated in their name. Their sequences are as follows and contain an *Eco*RI site and three additional 5′ residues: pEOΔ500 (B101: 5′ATAGAATTCACAGCCAAGACCAAGCG3′), pEOΔ517 (EKO1: 5′ATAGAATTCGCTGAGATACCTCAGG3′), pEOΔ556 (EKO2: 5′ATAGAATTCGTACCAACGGACCCTCGTG3′), pEOΔ567 (EKO3: 5′ATAGAATTCGGAGCCATGAAGGAATTTG3′), pEOΔ581 (EKO4: 5′ATA GAA TTCAAC A A C T C C G A G TCT3′), pEOΔ599 (EKO5: 5′ATAGAATTCGAGATCGCAAATAAG3′. See Table 1). Two other mutants, pEO15 and pEOΔ18, were made with PCR using B101 and B102 as primers and pB1PK15 and pB1PK18 as templates (Table 1, Kroner *et al.,* 1990). The pPK18 construct deletes 49 residues at the 1a C-terminus. pEOΔ567 through pEOΔ599 were sequenced (U.S. Biochemical Sequenase kit version 2.0 with terminal deoxynucleotidyl transferase; DeShazer *et al.,* 1994) and shown to be in the correct translational frame. In addition, the *in vitro* translation products of the PCR-generated mutants were all of the expected sizes.

Electroporation of yeast cells

A modification of the method described by Becker and Guarente (1991) was used. Yeast strain Y835 (Lys::*lexA*op-*HIS*3, *ura*::*lexA*op-*lacZ*, *trp*1-901, *leu* 2-3, -112, *ade*2, Δ*gal*14, Δ*gal*80) was grown to an OD_{600} of 1.0–1.5. Two hundred milliliters of cells was harvested by centrifugation at 2000 *g* for 5 min at 4°. All subsequent procedures were performed at 4° using ice-cold solutions. The cells were washed twice with water and once with 1 *M* sorbitol (harvesting between each wash), and then the cells were resuspended in 200 μl of 1 *M* sorbitol. Aliquots of the cells (40 μl) were then mixed with the appropriate plasmid DNAs and incubated on ice for 5 min. Electroporations (1.5 kV, 25μF, 200) were performed in a chilled 0.2-cm sterile cuvette using a Bio-Rad Gene Pulser. Immediately following the pulse, 1.0 ml of 1 *M* sorbitol was added to the cells, which were then plated on an appropriate synthetic medium (Sherman, 1991). Usually 1 μl of such a standard miniprep yielded 500–1000 transformants.

Detection of *in vivo* interaction in yeast

After 3 days of growth, the yeast transformants were assayed qualitatively for the production of β-galactosidase with the filter colony lift assay of Chevray and Nathans (1992). A Protran BA85 0.45-μm circular nitrocellulose filter was placed onto a plate of yeast transformants, and the position of the filter was marked with five needle stabs through the filter into the agar. Afterward the filter was placed in liquid nitrogen for approximately 10 sec and then transferred to a plastic tray containing Whatman paper soaked with Z buffer (1× concentration, 0.06 *M* $Na_2HPO_4 \cdot 7H_2O$, 0.04 *M* $NaH_2PO_4 \cdot H_2O$, 0.01 *M* KCl, 0.001 *M* $MgSO_4 \cdot 7H_2O$, pH 7.0, 0.05 *M* ß-mercaptoethanol, and 1 mg/ml X-Gal). Usually within 1 hr at 30°, the colonies containing ß-galactosidase turned dark blue.

Transformed strains were also assayed quantitatively for the production of ß-galactosidase with an ONPG (*O*-nitrophenyl-galactoside, Sigma) enzymatic assay of specific activity. Yeast extracts were prepared by scraping yeast cells (100–200μg) from plates and transferring them to a 1.5-ml Eppendorf tube. The cells were then washed twice with ice-cold water and harvested at 2000 *g* for 1 min in a microcentrifuge between washes. The washed pellet was then resuspended in 200 μl of Zbuffer with protease inhibitors (1× concentration, 0.06 *M* $Na_2HPO_4 \cdot 7H_2O$, 0.04 *M* $NaH_2PO_4 \cdot H_2O$, 0.01 *M* KCl, 0.001 *M* $MgSO_4 \cdot 7H_2O$, pH 7.0, 0.05 *M* ß-mercaptoethanol, 33.4 μg/ml PMSF (phenylmethylsulfonyl fluoride), 3.4 μg/ml leupeptin). Acid-washed and autoclaved glass beads were added to 80% of the total volume of the yeast suspension, and the mixture was vortexed for 3 min at full speed at 4°. The mixture was then microcentrifuged at 14 *g* for 15 min at 4°. The supernatant was used directly in the β-galactosidase activity (Ausubel *et al.,* 1994) and Bradford (1976) assays. BSA was used as a protein concentration standard in the Bradford assay. ß-Galactosidase specific activities were calculated as μmol of ONPG hydrolyzed/min/mg protein.

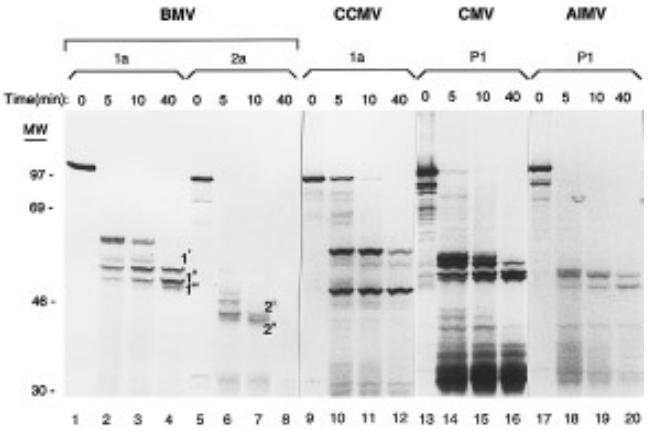

FIG. 1. Results of limited protease treatment of the helicase-like proteins of four tripartite RNA viruses. For comparison, the papain digestion pattern of BMV 2a is also included. The helicase-like proteins (1a or P1) of BMV, CCMV, CMV, and AlMV were *in vitro* transcribed and translated in rabbit reticulocyte lysates in the presence of [^{35}S]-methionine and then digested with papain as detailed under Materials and Methods. Aliquots of the digestion reactions were collected at 5, 10, and 40 min as indicated above each lane. The amount of material in the lanes for these time points represents 2.5 μl of the original translation reaction, while the material in the 0 min lane contains 1 μl of the original translation reaction. The positions of protein molecular mass markers (Rainbow markers, Amersham) and their sizes in kilodaltons are indicated to the left of the fluorogram. Within lane 4, the major protease-resistant bands (1′, 1″, and 1‴) of the BMV 1a after digestion for 40 min are indicated. The two major protease-resistant bands in 2a are labeled 2′ and 2′.

RESULTS

Protease-resistant domains are present in the helicase-like replication proteins of several tripartite RNA viruses

Based on sequence comparisons, all of the tripartite RNA viruses discussed in this paper encode proteins containing methyltransferase-like and helicase-like domains (Ahlquist *et al.,* 1985; Haseloff *et al.,* 1984). However, the nature of the structures corresponding to these sequences has not been demonstrated. *In vitro*- translated 1a binds to 2a in the same manner as *in vivo*-translated 1a (Kao *et al.,* 1992) and can be used as substrate for structural analyses. To examine structural features within BMV 1a, we performed limited digestion studies with papain, a low specificity endoproteinase that tends to cleave after phenylalanine, arginine, or lysine residues (Baker and Drenth, 1987). Papain digests globular domains at a much slower rate than nonglobular domains within the same molecule. This has been shown in numerous proteins, including the bacteriophage λ repressor and immunoglobulin G (IgG) proteins (Pabo *et al.,* 1979; Parham, 1986). Papain treatment of BMV 1a at several time points generated bands of between 50 and 65 kDa (Fig. 1, lanes 1–4). However, by the 40-min time point, only three major 50- to 55-kDa species remained (designated 1′, 1″, and 1‴; Fig. 1, lane 4). These persisting bands disappeared when translation reactions were treated with ionic detergents, when heated to 50° prior to papain digestion, or when 100-fold more enzyme was used (data not shown). In addition, the BMV 2a protein was much more sensitive than 1a to papain and was completely digested by 40 min (Fig. 1, lanes 5–8).

To determine whether such protease-resistant polypeptides were common to the replication proteins of other tripartite RNA viruses or were unique features of BMV, we translated the corresponding proteins of CCMV, CMV, and AlMV and performed papain digestions at 30° over time. Treatment of the helicase-like proteins of CCMV (Fig. 1, lanes 9–12), CMV (lanes 13–16), and AlMV (lanes 17–20) resulted in one or more major protease-resistant bands of ca. 50 kDa, similar to those of BMV 1a.

Stability of protease-resistant products of BMV 1a during time course experiments

To characterize further the kinetics of protease digestion, BMV 1a was treated for intervals ranging from 2 min to 16 hr (Fig. 2). At each time point an aliquot was removed and the reaction stopped by the addition of 2× Laemmli sample buffer. The samples were kept frozen on dry ice until all time points were collected. Of the three major 1a digestion products, band 1′ was apparent at the earliest (2 min) time point, while 1″ and 1‴ increased in abundance at some of the later times. The latter two bands represent the most stable core of the protease-resistant domain and some of the larger partial digestion products present at earlier times were eventually cleaved to yield the 1″ and 1‴ bands. All of these bands also persisted beyond the 40-min digestion time.

From the time course and subsequent experiments, the molar yields of the protease digestion fragments were calculated from their radioactivity by taking into

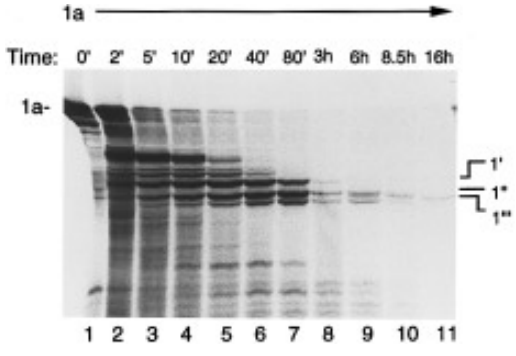

FIG. 2. Time course of digestion of *in vitro*-translated and [^{35}S]-methionine-labeled BMV 1a protein. Aliquots of the digestion reaction were terminated between 2 min and 16 hr as indicated above each lane. The undigested 1a polypeptide was loaded in lane 1 and its migration position is indicated to the left. The positions of the 1′, 1″, and 1‴ bands are indicated to the right of the fluorogram.

account the proportion of labeled methionine residues in the protected region versus the full-length protein. The amounts of radiolabel in the undigested material and the papain-resistant bands were determined using a digital imaging system. After normalizing for the distribution of methionine residues and the volume of reticulocyte lysate used and averaging several experiments, approximately 30% of the *in vitro*-translated wt 1a molecules were found to persist collectively in the form of bands 1′, 1″, and 1‴ after 40 min digestion at 30°.

Effects of small insertions on the BMV 1a protease-protected domain

Many two- or three-amino-acid insertions in the 1a protein previously have been constructed and analyzed for their ability to support replication in protoplasts (Kroner *et al.,* 1990) and their ability to bind wt 2a in a coimmunoprecipitation assay (Kao and Ahlquist, 1992). The location and phenotypes of these insertions are detailed in Table 1 and Fig. 3A. We analyzed the effects of these 19 mutations on the appearance of the major protease-resistant bands. In these translations, a uniform but faint spurious band of approximately 48 kDa (position denoted with an asterisk) was observed in prolonged exposures of all samples, whether digested or undigested. This band, which may arise from the plasmid vector containing the 1a gene, serves as a convenient internal protease-resistant control. Within the methyltransferase-like N-terminus, none of the nine insertions affected the protease-resistant bands (Fig. 3B, lanes 4–9, and results not shown). In the putative hinge between the two conserved domains, limited digestion of the PK4 and PK2 polypeptides yielded digestion patterns identical to wildtype 1a (data not shown), while the lethal mutant PK16 was completely degraded to fragments smaller than 20 kDa within 5 min (Fig. 3B, lanes 10–12).

Changes in or adjacent to the helicase-like domain can disrupt the protease-resistant region. Within the helicase-like domain, two mutations which do not affect RNA replication *in vivo,* PK14 and PK21, were resistant to papain digestion (Fig. 3B, lane 13–15, and data not shown), while three lethal insertion mutants, PK17 (lanes 19–21), PK20 (lanes 22–24), and PK18 (lanes 25–27), resulted in complete degradation of the mutant protein. PK15 is interesting because it retains the protease-resistant structure and the ability to bind to 2a *in vitro,* but cannot support RNA replication *in vivo*.

The PK19 insertion mutation renders 1a temperature sensitive for *in vivo* replication and *in vitro* binding to wt 2a (Kao *et al.,* 1992; Kroner *et al.,* 1990). To examine whether the temperature of synthesis affects the protease-resistant structure in PK19, wildtype 1a and PK19 were translated *in vitro* for 2 hr at either 24° or 32°, and then translations were terminated by treatment with Rnase A. After protease digestions at 24°, the abundance of protease-resistant bands was quantitated by laser densitometry and normalized for the amount of starting material. Wildtype 1a protein synthesized at 24° or 32° produced the usual complement of protease-resistant bands 1′, 1″, and 1‴ (Fig. 4, lanes 1–8), as did PK19 synthesized at 24° (Fig. 4, lanes 13–16). However, PK19 synthesized at 32° yielded fourfold less resistant bands at the 5-min time points than did wt 1a synthesized at 32° (Fig. 4, lanes 9–12). As a control, PK17 was sensitive to digestion by papain whether it was synthesized at 24 or 30° (Fig. 4, lanes 17–20). Therefore, the insertion in PK19 appears to have a temperature-dependent effect on the folding of the nascent polypeptide.

Further mapping of the 1a helicase-like region

The results from the 1a insertion mutations indicate that the integrity of the C-terminal helicase-like portion of 1a is important for the production and survival of protease-resistant bands 1′, 1″, and 1‴. To examine the 1a C-terminus further, we cleaved plasmids pPK18 and pPK20 (Fig. 5A) at the unique *Bam*HI restriction sites created by their linker-insertion mutations following nucleotides 2810 and 2788, respectively. Subsequent translations resulted in the polypeptides PK18/*Bam*HI and PK20/*Bam*HI that are missing the C-terminal 49 and 56 residues, respectively. These two truncated polypeptides were completely digested within the earliest sample time (2 min; data not shown). We also determined the effects of N-terminal 1a deletions on the protease digestion pattern. Digestion of 1a-Δ303 and 1a-Δ502 (Fig. 5A) with papain resulted in the usual complement of resistant bands found in wt 1a (Fig. 5B, lanes 1–4 and 12–14, and data not shown). The protected region thus lies completely within the C-terminal helicase-like portion of 1a. Furthermore, trypsin digestion of wt 1a and 1a-Δ502 yielded resistant products of similar sizes (Fig. 5, lanes 5–11). These results confirm that the resistant products are derived from the C-terminus of full-length 1a and demonstrate that the detection of a protease-resistant domain is not solely dependent upon the use of papain.

To map the papain cut sites that yielded the 1′, 1″, and 1‴ bands, a nested series of deletions that would remove 1a residues 2–517, 2–556, 2–567, 2–581, and 2–599 was constructed. The resulting proteins, 1a-Δ517, 1a-Δ556, 1a-Δ567, 1a-Δ581, and 1a-Δ599, were translated *in vitro* and treated with papain. 1a-Δ517 generated the 1′, 1″, and 1‴ bands, but 1a-Δ556 retained only the 1‴ band. The 1a-Δ567 polypeptide, which is slightly smaller than 1‴, was degraded by the 5-min time point (Fig. 5, lanes 23–26). In addition, 1a-Δ581 and 1a-Δ599 were sensitive to papain (data not shown). Therefore, the 1′ and 1″ bands have ends that lie between amino acids 518 and 555, and the end of the 1‴ band lies close to

TABLE 1

Summary of BMV Plasmids, Their Encoded Proteins, and Relevant Characteristics

Plasmid[a]	Encoded polypeptide	Amino acids inserted or deleted[b]	*In vivo* phenotype[c]	*In vitro* binding to 2a[d]	Protease-resistant structure[e]
pB1TP3	wt 1a	None	WT	+	+
1a insertion mutations					
pB1PK2	PK2	D-P (507)	WT	+	+
pB1PK3	PK3	G-S (5)	−	+	+
pB1PK4	PK4	G-S (492)	TS	+	+
pB1PK5	PK5	G-S (207)	WT	+	+
pB1PK6	PK6	G-S (95)	−	+	+
pB1PK7	PK7	G-S (239)	−	+	+
pB1PK9	PK9	G-S (154)	WT	+	+
pB1PK10	PK10	G-P (198)	−	+	+
pB1PK11	PK11	G-P (203)	−	+	+
pB1PK12	PK12	W-A-H (311)	−	+	+
pB1PK13	PK13	W-A-H (403)	−	+	+
pB1PK14	PK14	G-P (556)	WT	+	+
pB1PK15	PK15	W-A-H (651)	−	+	+
pB1PK16	PK16	G-P-T (464)	−	−	−
pB1PK17	PK17	D-P (869)	−	−	−
pB1PK18	PK18	G-S (912)	−	−	−
pB1PK19	PK19	G-S (670)	TS	TS	TS
pB1PK20	PK20	D-P (905)	−	−	−
pB1PK21	PK21	D-P (542)	WT	+	+
1a deletion mutations					
pla-Δ303	1a-Δ303	2-303	N.D.[f]	+	+
pla-Δ502	1a-Δ502	2-502	N.D.	+	+
pla-Δ517	1a-Δ517	2-517	N.D.	N.D.	+
pla-Δ556	1a-Δ566	2-556	N.D.	N.D.	+
pla-Δ567	1a-Δ567	2-567	N.D.	N.D.	−
pla-Δ581	1a-Δ581	2-581	N.D.	N.D.	−
pla-Δ599	1a-Δ599	2-599	N.D.	N.D.	−
Two-hybrid plasmids					
pEOΔ500	LexA-1aΔ500	2-500	W[g]		+
pEOΔ517	LexA-1aΔ517	2-517	W		+
pEOΔ556	LexA-1aΔ556	2-556	W		+
pEOΔ567	LexA-1aΔ567	2-567	W		+
pEOΔ581	LexA-1aΔ581	2-581	W		−
pEOΔ599	LexA-1aΔ599	2-599	W		−
pEO15	LexA-15	W-A-H(651)	W		−
pEOΔ18	LexA-18/BamHI	912-961	W		−
pGAD-2N	GAL4−2aN fusion	1-140[h]	W		−

[a] pB1TP3 was originally described by Janda *et al.* (1987). Plasmids in the PK insertion series were described by Kroner *et al.* (1990). The pla-Δ303 and pla-Δ502 plasmids were described by Kao and Ahlquist (1992), and the remainder of the deletion mutants were constructed as described under Materials and Methods.

[b] The insertions in PK2-PK21 occur directly after the indicated 1a amino acid number (in parentheses). The inserted amino acids are indicated by the standard one-letter codes. For deletion mutations, the numbers of the first and last amino acids that were deleted are shown.

[c] Ability of the mutant 1a gene to support BMV RNA replication in barley protoplasts inoculated with wt RNA2 and RNA3, as determined by Kroner *et al.* (1990). WT, RNA replication at 18, 24, and 35° was equivalent to that of wt infection; TS, RNA accumulation at 35° was markedly reduced relative to that at 24°; −, no RNA accumulation at 18, 24 or 35°.

[d] Ability of the mutant protein to bind wt 2a proteins cotranslated in reticulocytes (Kao and Ahlquist, 1992). +, binding similar to wt 1a; −, no detectable binding.

[e] Ability of each mutant 1a protein to be cleaved by papain and yield the same pattern of major protease resistance fragments as the WT construct.

[f] N.D., Not Determined.

[g] Color of yeast colonies transformed with the indicated single plasmid. W, white.

[h] Amino acids 1−140 of the 2a N-terminus fused downstream of the GAL4 activation domain.

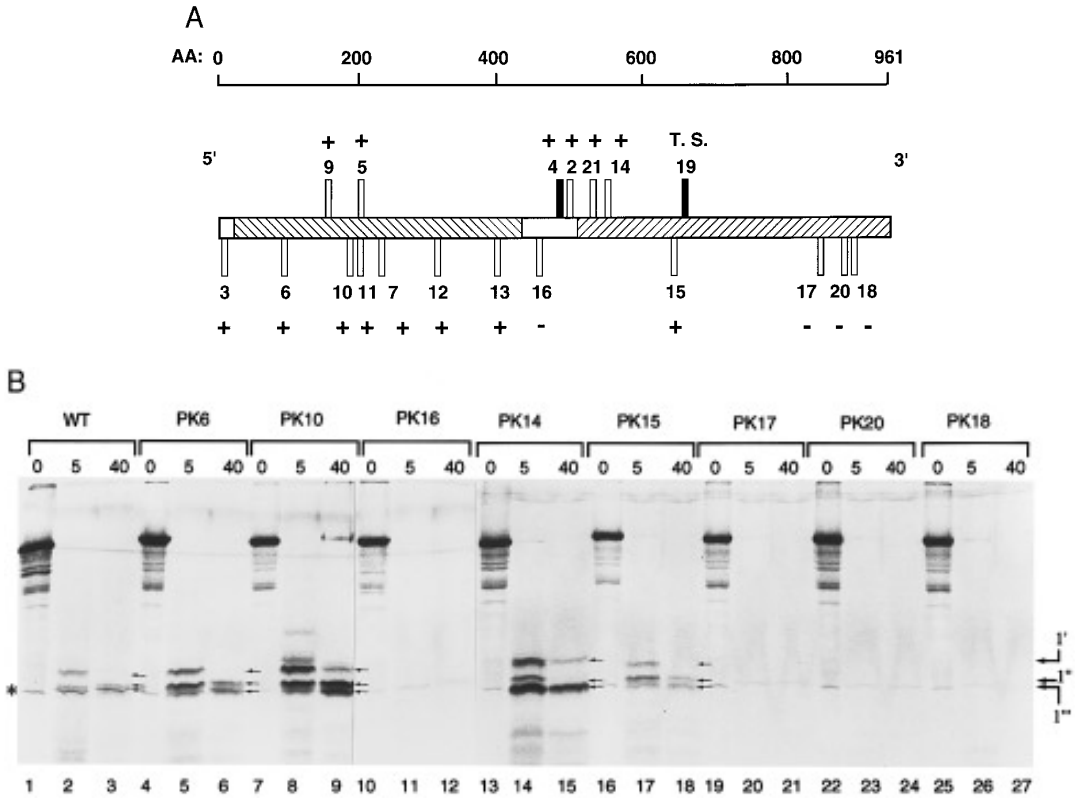

FIG. 3. Effects of two- and three-amino-acid insertions in the 1a polypeptide on the protease-resistant bands. (A) Locations of the insertions and summary of their effects on digestion by papain. The scale at the top shows amino acid (AA) position within 1a. The methyltransferase-like and helicase-like domains are represented by the hatched areas in the N- and C-termini, respectively. The unshaded flanking and central areas represent residues that are less conserved relative to analogous proteins of other plus-strand RNA viruses. A + or − indicates the presence or absence of the usual protease-resistant bands after papain treatment (see B). Those insertion mutations that can support RNA replication in protoplasts are shown above the bar representing the 1a polypeptide (Kroner *et al.,* 1990). The positions of insertions that diminished *in vivo* RNA replication below detection limits are shown projected below the 1a polypeptide. Mutants PK4 and PK19, denoted by black bars, are temperature-sensitive. (B) Fluorogram of the papain digestion pattern of representative PK insertion mutants. The polypeptides were digested for 0, 5, or 40 min as indicated above each lane. The locations of protease-resistant bands 1′, 1″, and 1‴, where present, are noted by arrows. An approximately 48-kDa band present both before and after the protease digestion is indicated by an asterisk (*) on the left.

residue 556. The digestion products of 1a-Δ556 were indistinguishable in mobility from those of the untreated protein and the wt-derived 1‴ band, but the protease was active in this reaction, as evidenced by the generation of fainter low-molecular-weight bands. To further verify the resistance of 1a-Δ556 to papain, we have cotranslated 1a-Δ556 with wt 2a and witnessed the usual rapid digestion of the 2a molecule in parallel with the persistence of 1a-Δ556 (data not shown).

Papain-resistant structures in the helicase-like domain of three tripartite RNA viruses

A closer examination of the BMV 1a amino acid sequence upstream of the papain cut sites in the center of the protein revealed a sequence rich in proline. BMV 1a contains 28 prolines, 9 of which are clustered between residues 514 and 560 (Fig. 6A). Similar proline-rich regions are present in the analogous helicase-like proteins of CCMV (Dzianott and Bujarski, 1991) and AlMV (Cornelissen *et al.,* 1983a). In CCMV 1a, 6 proline residues are found between amino acids 531 and 560. In AlMV P1, 6 prolines are present in the 12 residues between residues 673 and 684 (Fig. 6A). To determine where the protease-resistant bands in CCMV and AlMV helicase-like proteins map with respect to these sequences, we expressed three N-terminal truncations: PCC1-2, which encodes CCMV 1a with a deletion of residues 2–548, and two encoding AlMV P1 truncations, PAl1-3 and PAl2-3, which are missing residues 2–520 and 2–675, respectively. PCC1-2 and PAl2-3 encode proteins that remove N-terminal residues up to and including a portion of the proline cluster (Fig. 6A). All three truncations were digested in parallel with their respective wt proteins (Fig. 6B). Wild-type CCMV 1a protein and PCC1-2 both yielded identically-sized products of approximately 48 kDa, co-migrating with untreated PCC1-2 (Fig. 6B, lanes 1–8). A slower-migrating band corresponding to the one observed in Fig. 1 (lanes 10–12) was visible in longer exposures of the 5-min digestion of wt CCMV 1a (Fig. 6B, lane 2).

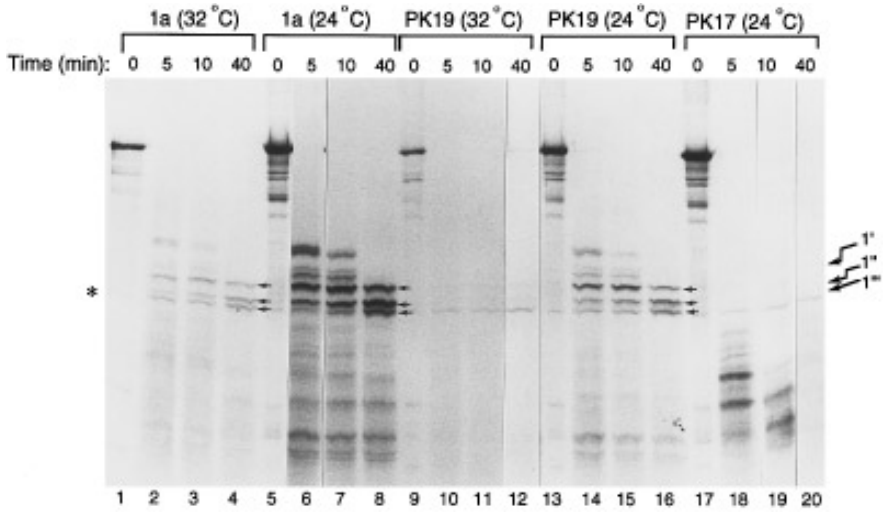

FIG. 4. Protease digestion pattern of 1a temperature-sensitive mutant PK19. The identity of the [^{35}S]methionine-labeled polypeptide being tested, the temperature of *in vitro* translation, and the time of digestion (in min) are indicated on the top of the fluorogram. Papain digestion of all samples was performed at 24°. Locations of the three major protease-resistant bands are denoted to the right of the fluorogram and are pointed out with arrows, when present. The position of the approximately 48-kDa band discussed in the Fig. 3 legend and in the text is denoted by the asterisk (*) on the left.

Digestion of wt AlMV P1 and its two N-terminally truncated derivatives yielded the two major protease-resistant species of approximately 45–48 kDa (lanes 9–20). The higher molecular weight band comigrated with untreated PAl2-3. Therefore, we conclude that the protease-resistant domains in CCMV and AlMV 1a proteins immediately follow proline clusters, just as in BMV 1a.

Interaction of BMV protein in yeast

To examine the interaction of BMV 1a and 2a *in vivo,* we used the two-hybrid system. Since BMV 1a and 2a proteins are functional in yeast and can direct the replication of a BMV replicon (Janda and Ahlquist, 1993), yeast is an ideal system for such analyses. Domains sufficient for 1a–2a interaction *in vitro* (Kao and Ahlquist, 1992) were fused in-frame to two-hybrid plasmids: the 1a-helicase-like domain (codons 500 to 961) was fused to the LexA protein in plasmid pEOΔ500 and the 2a N-terminal 140 amino acids were fused in-frame to the GAL4 transcription activation domain to generate pGAD-2N. When present together in the same yeast cell, these plasmids activated the expression of the reporter gene β-galactosidase to yield blue colonies, thus indicating a physical interaction between the BMV-derived proteins *in vivo*. When the DNA encoding the 2a N-terminus is in the opposite orientation with regard to GAL4, the construct is not able to induce β-galactosidase activity in the presence of pEOΔ500. In yeasts harboring pGAD-2N and pEOΔ500, quantitative measurements revealed greater than 100-fold higher β-galactosidase activity than in yeast strains lacking either one or both of the two plasmids (Table 2). Curing the blue colonies of either the 1a or the 2a two-hybrid plasmids caused the loss of β-galactosidase activity. Likewise, colonies containing pEOΔ500 and pEE5 (Chien *et al.,* 1991), which express a protein unrelated to BMV, do not have β-galactosidase activity.

The interactions between domains within 1a and 2a in yeast have requirements similiar to those in plant protoplasts. Deletions of amino acids 2–50 in the 2a N-terminus reduced, but did not abolish, RNA replication in protoplasts. More extensive deletions did abolish RNA replication. In yeast, plasmid pGAD-2NΔ2-40, which deletes amino acids 2–40, is still capable of inducing β-galactosidase activity in the presence of pEOΔ500. Deletions of amino acids 2–70 or 2–90 prevents this interaction (Table 2). Thus, the 1a–2a interaction in yeasts occurs in a manner consistent with results from previous coimmunoprecipitation and *in vivo* RNA replication assays (Kao and Ahlquist, 1992; Traynor *et al.,* 1991).

In order to examine whether the 1a protease-resistant structure plays a role in the interaction with 2a in yeast, we fused several of the 1a N-terminal truncations with LexA and transformed them into yeast with pGAD-2N (Table 2). All plasmids encoding 1a fusion proteins were unable to induce significant β-galactosidase activity in the absence of pGAD-2N. Proteins LexA-1aΔ500 through LexA-1aΔ567 retained the ability to interact with the N-terminus of 2a *in vivo,* while LexA-1aΔ581 and LexA-1aΔ599 did not. An insertion of 3 amino acids into the helicase-like portion of 1a, pEO15, had only a minor affect on the interaction with pGAD-2N (Table 2). This correlates with the *in vitro* protease resistance of PK15 (Kao, 1992b; Table 2). A C-terminal deletion which removes

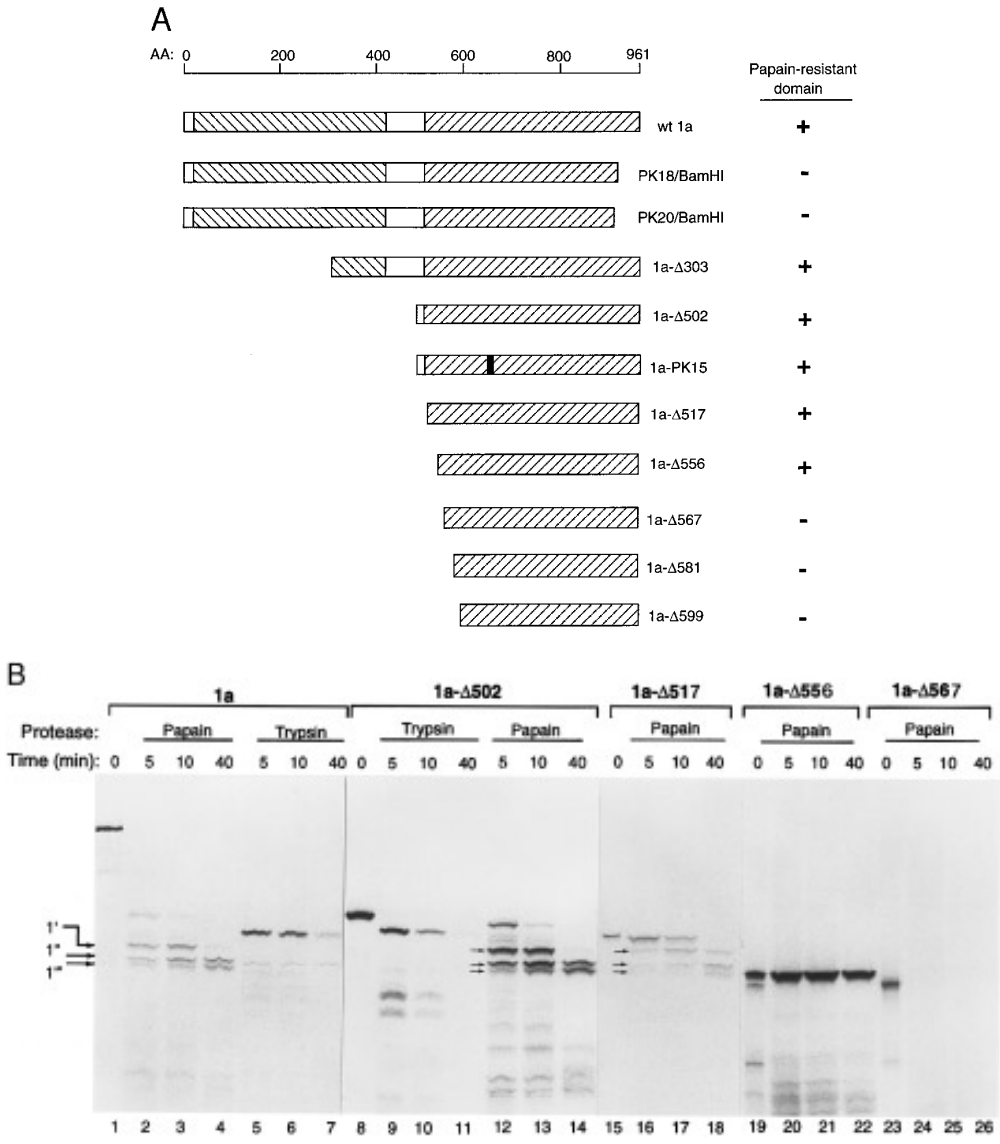

FIG. 5. Further mapping of the protease-resistant bands in BMV 1a. (A) Schematic of the 1a deletion mutations that were tested for protease-resistant digestion pattern. The scale at the top shows amino acid (AA) position within 1a. The various domains in 1a are illustrated as described in the legend to Fig. 3A. PK18/BamHI and PK20/BamHI contained C-terminal truncations that were generated by digestion of the 1a gene at the unique *Bam*HI sites in pPK18 and pPK20 (Table 1) followed by *in vitro* transcription and translation. Whether or not the truncated polypeptide generated protease-resistant bands after digestion by papain is indicated by + or −, respectively. (B) Fluorogram of the protease digestion pattern of selected 1a deletion proteins. The identity of the polypeptide being tested and the time of digestion (in min) are indicated above the lanes. Digestions of *in vitro*-translated 1a proteins was with either papain or trypsin as indicated. The positions of the three major papain-resistant bands are denoted to the left of the fluorogram and within the lanes of digestion products.

the last 49 amino acids, pEOΔ18, abolished interaction with the 2a protein. In the protease digestion assay, this truncation rendered 1a sensitive to papain. The only truncated protein which was unstable *in vitro* but which could interact with 2a in yeast was LexA-1aΔ567 (Table 2). To determine whether this difference was the result of adding LexA sequence to 1aΔ567, papain digestions were performed on several of the fusion proteins. Again, LexA-Δ517 was resistant to papain digestion, while LexA-Δ567 and LexA-Δ580 were sensitive. The result was the same even when the digestions were performed at 15° (data not shown). We attribute the differential stability of truncation 1aΔ567 to an enhanced stability *in vivo*.

DISCUSSION

Limited proteolysis studies have been used to better understand protein structure and function. Several eukaryotic transcription factors (for examples, see Boulanger *et al.,* 1989, and Lieberman *et al.,* 1991) can

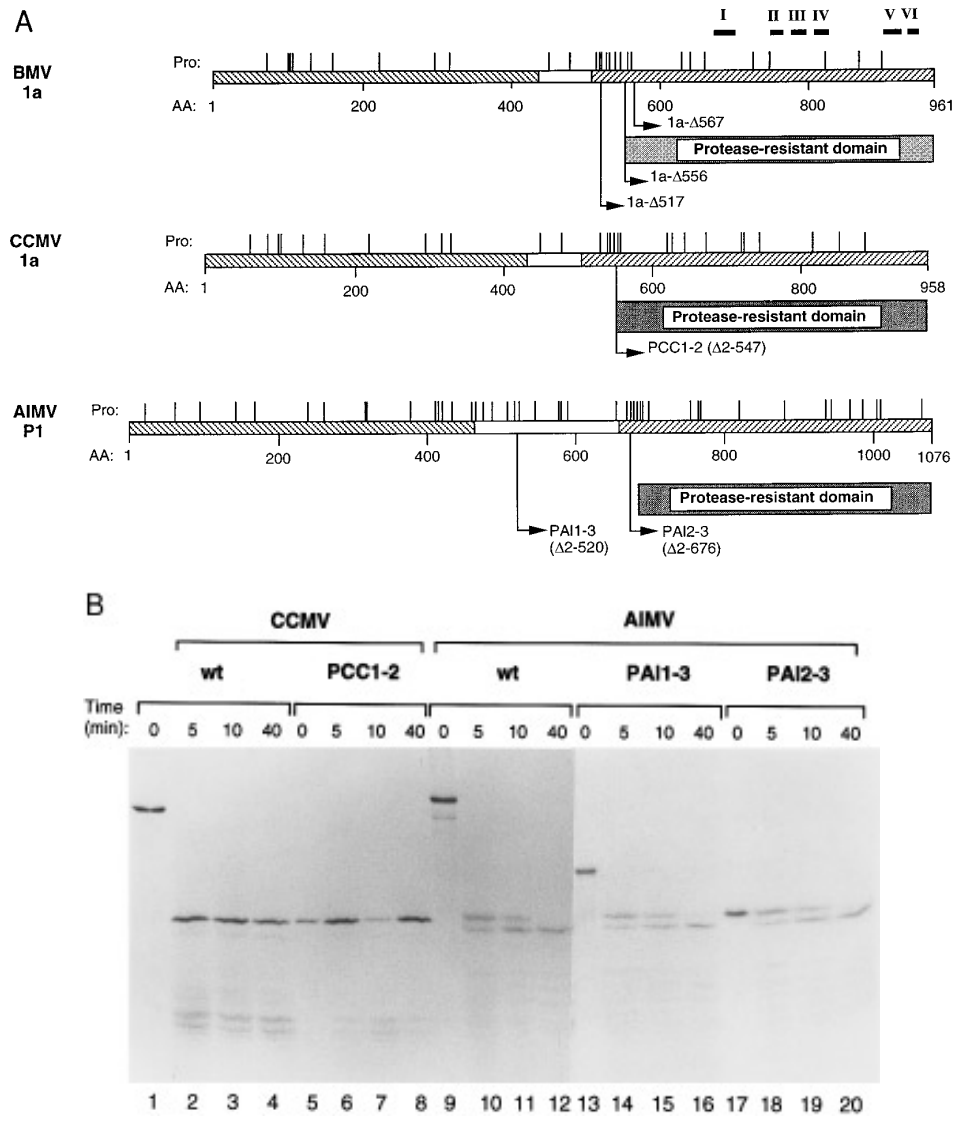

FIG. 6. Conservation in location of the protease-resistant region in the helicase-like proteins of three tripartite RNA viruses. (A) Distribution of proline residues in the BMV, CCMV, and AlMV helicase-like proteins and the locations of the protease-resistant domains. The location of each proline residue is denoted by a vertical line above the bar representing the helicase-like polypeptide. The N-terminal methyltransferase-like and C-terminal helicase-like domains within each polypeptide are denoted by cross-hatching. A scale of the polypeptide in amino acid (AA) residues is below each map. The N-terminal limits of the deletions tested by papain digestion are indicated by arrows under the bar representing the 1a or P1 polypeptides. The approximate limits of the protease-resistant regions are indicated by the shaded bar under the C-terminal half of each polypeptide. The locations of the six conserved motifs found in common with ATP-dependent helicases (Hodgman, 1988; Kroner *et al.,* 1990) are indicated above the BMV 1a polypeptide. (B) Effect of deletions in the CCMV and AIMV proteins on the protease digestion pattern. The wt helicase-like proteins and their truncated derivatives used in these protease-digestion studies are indicated at the top of the fluorogram. Times of digestion (in min) are indicated above each lane. Lanes at time 0 contained untreated *in vitro* translation product. In lane 7, some of the sample was lost during processing before loading of the 8% polyacrylamide–SDS gel.

be partially digested with protease and retain some function, such as binding to DNA. Also, *Escherichia. coli* DNA polymerase I is differentially sensitive to proteases, resulting in the Klenow fragment (Jacobsen *et al.,* 1974). In this report we found that a distinct protease-resistant structure exists within the helicase-like RNA replication proteins of four tripartite viruses. Furthermore, we demonstrated in the BMV 1a protein, the integrity of this structure *in vitro* is closely correlated with functions *in vivo,* including the ability to interact with BMV 2a protein.

For BMV 1a, we mapped a protease-resistant structure from residue 556 to the C-terminus of the 1a protein (Fig. 5). At the C-terminus a deletion of the last 49 residues abolished the protease-resistant bands, indicating that the C-terminal residues contribute to stability of the domain. In fact, it appears that most or all of the extreme C-terminal residues reside within the protease-resistant

TABLE 2

Summary of the Interaction between 1a and 2a in the Two-Hybrid System

Yeast strains	Appearance of blue colonies in filter assay	β-Galactosidase specific activity			
		Expt 1[a]	Expt 2	Expt 3	Expt 4
Y835 with					
no plasmid	W	0	1.4	1.3	0
pEOΔ500[b]	W	2.3	2.9	1.4	0
pEE5	W	N.D.[c]	N.D.	1.3	N.D.
pGAD-2N	W	0	1.6	1.9	0
pGAD-Δ2-40	W	2.7	2.4		
pGAD-Δ2-70	W	2.0	2.9		
pGAD-Δ2-90	W	1.1	0.3		
Y835 with pEOΔ500 and					
pGAD-2N	B	223	253		
pGAD-2NΔ2-40	B	79.6	55.5		
pGAD-2NΔ2-70	W	4.7	5.0		
pGAD-2NΔ2-90	W	1.3	1.1		
Y835 with pGAD-2N and					
pEOΔ500	B			529	232
pEO15	B			357	208
pEOΔ18	W			4.1	0
pEOΔ517	B			376	179
pEOΔ556	B			198	259
pEOΔ567	B			144	196
pEOΔ581	W			4.2	0
pEOΔ599	W			6.9	0

[a] Results from each experiment were the average of two independent assays. Specific activity is calculated as μmol of *O*-nitrophenyl galactoside hydrolyzed per minute per mg of protein.

[b] Description of the plasmids are in Table 1.

[c] Not determined.

region, since truncation 1a-Δ556 was not detectably digested by papain, and comigrated with the protease-resistant 1‴ fragment of wt 1a both before and after protease treatment (Fig. 5B).

Several independent results suggest that the protease-resistant structure in the helicase-like domain is needed for RNA replication. First, all small insertion mutations (PK16, PK17, PK18, and PK20 Kroner *et al.,* 1990) that disrupt this protease-resistant structure were unable to support BMV RNA replication *in vivo* (Fig. 3 and Table 1; see also Kroner *et al.,* 1990). Second, the temperature-sensitive nature of the protease-resistant domain in PK19 is correlated with the temperature-sensitive nature of RNA replication *in vivo*.

The protease-resistant structure appears to be needed to bind the polymerase-like 2a protein, a phenomenon which is quite possibly requisite to RNA replication. However, the ability of PK15 to maintain binding to 2a, but not RNA replication, indicated that binding is only one of the requirements for RNA replication. The four mutations in 1a (PK16, PK17, PK18, and PK20) which prevent 1a–2a interaction lack the protease-resistant structure, while all five mutations that do allow 1a–2a interaction (PK4, PK2, PK21, PK14, and PK15) retain the protease-resistant structure. In the two-hybrid system, we observed a good correlation between the presence of the protease-resistant domain and the ability to interact with 2a. The only exception to the correlation was the truncation 1a-Δ567, which was sensitive to digestion by papain *in vitro,* but could interact with the N-terminus of 2a in yeast. Since fusion to LexA does not appear to contribute to greater resistance to protease digestion *in vitro,* the deletion in 1a-Δ567 may be stabilized by molecules present in the cellular milieu. We note that 1a-Δ581 and other more severe deletions are both sensitive to papain *in vitro* and unable to interact with the 2a N-terminus in yeast.

Two of the insertion mutations in 1a deserve comment. First, PK16, while lying outside the minimal protease-resistant domain, nonetheless made the resultant protein sensitive to papain digestion. PK16 may have had a global effect on protein folding. Consistent with this hypothesis, it also affected binding to 2a even though it is not present in minimal domains required for interaction with 2a (Kao *et al.,* 1992). Mutation PK15 was also interesting because it was not detectably affected by papain digestion *in vitro,* can interact with the 2a N-terminus in

yeast, but was defective for replication *in vivo* (Kroner *et al.,* 1990). The insertion in PK15 is located just N-terminal to the conserved helicase motifs and may interfere with enzymatic activity of 1a rather than cause a gross unfolding of the helicase-like domain.

Several tripartite RNA viruses have a similar protease-resistant region in their helicase-like protein. The protease-resistant structure in BMV and CCMV 1a and in the analogous AlMV P1 protein consistently mapped to their respective helicase-like C-termini and encompassed all six previously recognized helicase sequence motifs in these proteins (Fig. 6). Moreover, all three protease-resistant structures occur immediately C-terminal to clusters of six to seven closely spaced proline residues. These prolines may represent areas of discontinuity in the structure of these proteins. The consistent arrangement, coupled with the results from proteolysis studies, provides further evidence for the conservation of structural organization in these proteins, which in turn, may relate to similar functional activities. The area encompassing the proline cluster of the BMV 1a protein is at least partially resilient to mutations since insertions PK14 and PK21 did not adversely affect RNA replication *in vivo* (Fig. 3A; Kroner *et al.,* 1990). The definition of a protease-resistant structure in the helicase-like domain of tripartite RNA viruses will be useful for future structural and functional studies of these proteins. Finally, while protease-resistant structures do indicate the presence of higher order folding, results from these studies do not imply that protease sensitivity indicates the lack of an organized structure.

ACKNOWLEDGMENTS

We thank P. Palukaitis for cDNAs of CMV and J. Bol for cDNAs of AlMV, Stan Fields and Steve Elledge for two-hybrid plasmids, R. Quadt for helpful suggestions on the manuscript, and L. Kao for her careful editing. This research was supported by the National Institute of Health under Public Service Grant GM35072 to P.A. Funding by National Science Foundation Plant Biology DIR9104366 to C.C.K. is acknowledged.

REFERENCES

Ahlquist, P. (1992). Bromovirus RNA replication and transcription. *Curr. Opin. Genet. Dev.* 2, 71–76.

Ahlquist, P., Strauss, E. G., Rice, C. M., Strauss, J. H., Haseloff, J., and Zimmern, D. (1985). Sindbis virus proteins nsP1 and nsP2 contain homology to nonstructural proteins from several RNA plant viruses. *J. Virol.* 53, 536–542.

Allison, R., Janda, M., and Ahlquist, P. (1988). Infectious *in vitro* transcripts from cowpea chlorotic mottle virus cDNA clones and exchange of individual components with brome mosaic virus. *J. Virol.* 62, 3581–3588.

Argos, P. (1988). A sequence motif in many polymerases. *Nucleic Acids Res.* 16, 9909–9916.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (Eds.) (1994). "Current Protocols in Molecular Biology." Wiley, New York.

Baker, E. N., and Drenth, J. (1987). *In* "Biological Macromolecules and Assemblies, Vol. 3, Active Sites of Enzymes" (F. A. Jurnakand A. McPherson, Eds.), pp. 314–367. Wiley, New York.

Becker, D. M., and Guarente, L. (1991). High-efficiency transformation of yeast by electroporation. *In* "Methods in Enzymology: Guide to Yeast Genetics and Molecular Biology" (C. Guthrie and G. R. Fink, Eds.), Vol. 194, Chap. 12. Academic Press, San Diego,CA.

Boulanger, P. A., L'Etoile, N. D., and Berk, A. J. (1989). A DNA-binding domain of human transcription factor IIIC2. *Nucleic Acids Res.* 17, 7761–7770.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.

Chien, C., Bartel, P. L., Sternglanz, R., and Fields, S. (1991). The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest. *Proc. Natl. Acad. Sci. USA* 88, 9578–9582.

Chevray, P. M., and Nathans, D. (1992). Protein interaction cloning in yeast: Identification of mammalian proteins that react with the leucine zipper of Jun. *Proc. Natl. Acad. Sci. USA* 89, 5789–5793.

Cornelissen, B. J. C., Brederode, F., Moormann, R. J. M., and Bol, J. F. (1983a). Complete nucleotide sequence of alfalfa mosaic virus RNA 1. *Nucleic Acids Res.* 11, 1253–1265.

Cornelissen, B. J. C., Brederode, F.,Veeneman, G. H., Van Boom, J. H., and Bol, J. F. (1983b). Complete nucleotide sequence of alfalfa mosaic virus RNA 1. *Nucleic Acids Res.* 11, 3019–3025.

DeShazer, D., Wood, G. E., and Friedman, R. L. (1994). Boiling eliminates artifact banding when sequencing double-stranded templates. *Biotechniques* 17, 288–290.

Dinant, S., Janda, M., Kroner, P., and Ahlquist, P. (1993). Bromovirus RNA replication and transcription require compatility between the polymerase-and helicase-like RNA synthesis proteins. *J. Virol.* 67, 7181–7189.

Dzianott, A. M., and Bujarski, J. J. (1991). The nucleotide sequence and genome organization of the RNA-1 segment in two bromoviruses: Broad bean mottle virus and cowpea chlorotic mottle virus. *Virology* 185, 553–562.

Evans, R. K., Haley, B. E., and Roth, D. A. (1985). Photoaffinity labeling of a viral induced protein from tobacco mosaic virus. Characterization of nucleotide binding properties. *J. Biol. Chem.* 260, 7800–7804.

French, R., Janda, M., and Ahlquist, P. (1986). Bacterial gene inserted in an engineered RNA virus: Efficient expression in monocotyledonous plants. *Science* 231, 1294–1297.

Goldbach, R., LeGall, O., and Wellink, J. (1991). Alpha-like viruses in plants. *Semin. Virol.* 2, 19–25.

Gorbalenya, A. E., Koonin, E. V., Donchenko, A. P., and Blinov, V. M. (1988). A novel superfamily of nucleotide triphosphate-binding motif containing proteins which are probably involved in duplex unwinding in DNA and RNA replication and recombination. *FEBS Lett.* 235, 16–24.

Haseloff, J., Goelet, P., Zimmern, D., Ahlquist, P., Dasgupta, R., and Kaesberg, P. (1984). Striking similarities in amino acid sequence among nonstructural proteins encoded by RNA viruses that have dissimilar genomic organization. *Proc. Natl. Acad. Sci. USA* 81, 4358–4362.

Hodgman, T. C. (1988). A new superfamily of replicative proteins. *Nature (London)* 333, 22–23. (Erratum 333:578).

Jacobsen, H., Klenow, H., and Overgaard-Hansen, K. (1974). The N-terminal amino acid sequence of DNA polymerase I from *Escherichia coli* and the large and small fragments obtained by a limited proteolysis. *Eur. J. Biochem.* 45, 623–627.

Janda, M., and Ahlquist, P. (1993). RNA-dependent replication, transcription, and persistence of brome mosaic virus RNA replicons in C. cerevisiae. *Cell* 72, 961–970.

Janda, M., French, R., and Ahlquist, P. (1987). High efficiency T7 polymerase synthesis of infectious RNA from cloned brome mosaic virus

cDNA and effects of the 5′ extensions on transcript infectivity. *Virology* 158, 259–262.

Kamer, G., and Argos, P. (1984). Primary structural comparison of RNA-dependent polymerases from plant, animal and bacterial viruses. *Nucleic Acids Res.* 12, 7269–7282.

Kao, C. C., and Ahlquist, P. (1992). Identification of the domains required for direct interaction of the helicase-like and polymerase-like RNA replication proteins of brome mosaic virus. *J. Virol.* 66, 7293–7302.

Kao, C. C., Quadt, R., Hershberger, R. P., and Ahlquist, P. (1992). Brome mosaic virus RNA replication proteins 1a and 2a form a complex *in vitro*. *J. Virol.* 66, 6322–6329.

Kiberstis, P. A., Loesch-Fries, L. S., and Hall, T. C. (1981). Viral protein synthesis in barley protoplasts inoculated with native and fractionated brome mosaic virus RNA. *Virology* 112, 804–808.

Kroner, P. A., Young, B. M., and Ahlquist. P. (1990). Analysis of the role of brome mosaic virus 1a protein domains in RNA replication, using linker insertion mutagenesis. *J. Virol.* 64, 6110–6120.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 277, 680–685.

Lane, L. C. (1981). Bromoviruses. *In* "Handbook of Plant Virus Infections and Comparative Diagnosis" (E. Kurstak, Ed.), pp. 333–376. Elsevier/North-Holland Press, Amsterdam.

Lieberman, P. M., Schmidt, M. C., Kao, C. C., and Berk, A. C. (1991). Two distinct domains in the yeast transcription factor IID and evidence for a TATA box-induced conformational change. *Mol. Cell. Biol.* 11, 63–74.

Mi, S., and Stollar, V. (1991). Expression of Sindbis virus nsP1 and methyltransferase activity in *Escherichia coli*. *Virology* 184, 423–427.

Pabo, C. O., Sauer, R. T, Sturtevant, J. M., and Ptashne, M. (1979). The lambda repressor contains two domains. *Proc. Natl. Acad. Sci. USA* 76, 1608–1612.

Parham, P. (1986). Preparation and purification of active fragments from monoclonal antibodies. In "Cellular Immunology" (D. M. Weir, Ed.),4th ed., Vol. 1, Chap. 14. Blackwell Sci, San Diego, CA.

Quadt, R., and Jaspars E. M. J. (1990). Purification and characterization of brome mosaic virus RNA-dependent RNA polymerase. *Virology* 178, 189–194.

Quadt, R., Verbeen, H. J. M., and Jaspars, E. M. J. (1988). Involvement of a nonstructural protein in the RNA synthesis of brome mosaic virus. *Virology* 165, 256–261.

Rizzo, T. M., and Palukaitis, P. (1989). Nucleotide sequence and evolutionary relationships of cucumber mosaic virus (CMV) strains: CMV RNA1. *J. Gen. Virol.* 70, 1–11.

Rizzo, T. M., and Palukaitis, P. (1988). Nucleotide sequence and evolutionary relationships of cucumber mosaic virus (CMV) strains: CMV RNA2. *J. Gen. Virol.* 69, 1777–1787.

Scheidel, L. M. and Stollar, V. (1991). Mutations that confer resistance to mycophenolic acid and ribavirin on Sindbis virus map to the nonstructural protein nsP1. *Virology* 181, 490–499.

Sherman, F. (1991). Getting started with yeast. *In* "Methods in Enzymology: Guide to Yeast Genetics and Molecular Biology" (C. Guthrie and G. R. Fink, Eds.), Vol 194, Chap. 1. Academic Press, San Diego, CA.

Traynor, P., Young, B. M., and Ahlquist, P. (1991). Deletion analysis of brome mosaic virus 2a protein: Effects on RNA replication and systemic spread. *J. Virol.* 65, 2807–2815.